Frontiers in Liver Transplantation

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

"Resuscitation" of marginal liver allografts for transplantation with machine perfusion technology

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Summary

As the rate of medically suitable donors remains relatively static worldwide, clinicians have looked to novel methods to meet the ever-growing demand of the liver transplant waiting lists worldwide. Accordingly, the transplant community has explored many strategies to offset this deficit. Advances in technology that target the *ex vivo* "preservation" period may help increase the donor pool by augmenting the utilization and improving the outcomes of marginal livers. Novel *ex vivo* techniques such as hypothermic, normothermic, and subnormothermic machine perfusion may be useful to "resuscitate" marginal organs by reducing ischemia/reperfusion injury. Moreover, other preservation techniques such as oxygen persufflation are explored as they may also have a role in improving function of "marginal" liver allografts. Currently, marginal livers are frequently discarded or can relegate the patient to early allograft dysfunction and primary non-function. Bench to bedside advances are rapidly emerging and hold promise for expanding liver transplantation access and improving outcomes.

Introduction

The advent of successful liver transplantation (LT) by Starzl *et al.* shifted the paradigm of hepatic failure as a terminal illness into a triumph of long-term meaningful survival [1,2]. However, the overwhelming success of LT has created a new dilemma in which the organ supply cannot meet the demand for LT. While there are approximately 6000 liver transplants performed each year in the United States, there are more than 11,000 patients added to the waiting list every year [3]. As such, waitlist mortality has become a central issue for the liver transplant community.

Early efforts to correct this disparity centered on the use of the MELD (model for end-stage liver disease) score based allocation. Interestingly, unlike end-stage renal disease, waitlist mortality does not correlate with waiting list time for patients with end-stage liver disease (ESLD) [4]. As such, the MELD score served to identify the sickest patients who had the highest risk of pre-transplant death and moved them to the "top of the list" [5].

Living donor liver transplantation (LDLT) has also arisen as a means to confront the growing disparity between supply and demand. The Adult-to-Adult Living Donor Liver Transplantation Cohort Study (A2ALL), a North American consortium of transplant centers, published excellent outcomes with LDLT [6]. This initial report and forth coming validation helped promote living donation as a viable option for patients looking to minimize their waitlist mortality risk [7]. However, LDLT while routine at many centers has not and will probably never significantly address the supply and demand disparities due to issues of donor safety and morbidity; not to mention the increased technical demands of LDLT.

Ten to fifteen percent of patients on the waiting list die annually before a liver graft becomes available [8,9]. An effort to reduce wait list mortality has included exploring other methods to improve access to transplant and reduce waiting time. There are also many patients who are underserved by the MELD score, and would benefit from early transplantation or will not achieve a high enough MELD score to receive an organ offer in regions with donor scarcity. Unfortunately, this can usually only be accomplished with the use of so-called "marginal grafts" or LDLT.

Expanding the donor pool by utilizing "marginal" grafts, which include extended criteria donor (ECD) and donor after cardiac death (DCD) livers appears to be the most logical answer. However, expansion of the donor pool with organs that have a higher failure rate seems irresponsible without concomitantly searching for novel interventions to improve the function of these ECD/DCD livers.

Our center has reported that transplantation of ECD livers improved patient survival and showed that the use of these marginal liver grafts maximizes donor utilization and increased access to liver transplantation [10]. Barshes *et al.* also

Keywords: Marginal liver; Liver; Transplantation; Perfusion; Machine preservation; Organ preservation; Extended criteria donor; Hypothermic; Normothermic; Subnormothermic.

Received 6 July 2013; received in revised form 13 April 2014; accepted 16 April 2014

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demonstrated that using ECD livers as defined by the New York State Department of Health Workgroup significantly reduced waiting list mortality [11,12]. As such, there has been a shift toward greater use of ECD livers. However, this utilization is not without problems and may lend itself to adverse outcomes.

The use of other types of livers, specifically DCD allografts by aggressive centers have also been proffered as a means to reduce waitlist mortality. However, recent trends showed progressively decreased utilization of DCD livers due to higher rates of ischemic cholangiopathy and graft loss [13]. The expanded use of marginal livers has also prompted the transplant community to confront the issue of preservation related ischemia/reperfusion injury (IRI). Techniques to ameliorate the clinical adverse effects of IRI have become a focus of research worldwide.

In an excellent review, Peralta *et al.* make the case that the consequences of the bimodal nature of IRI is perhaps the most important factor that influences graft dysfunction after transplantation [14]. Initially, the resultant lack of a terminal electron depot due to hypoxia leads to disruption of aerobic respiration and subsequent cellular metabolic disarray. Reperfusion further damages the organ via oxygen free radicalization and sheer stress injury to ischemically challenged sinusoidal endothelial cells. Van Golen *et al.* also illustrate the inciting events that evoke an inflammatory cascade in organs subject to IRI [15]. At the hepatocellular level, deprivation of oxygen promotes the release of damage associated molecular patterns (DAMPs) like HMGB-1, which leads to activation of Kupffer and dendritic cells. Reperfusion exacerbates IRI when reflow facilitates contact between these antigen presenting cells (APCs) and primed T-cells, neutrophils, and monocytes. This concept of the inflammatory response in IRI is theorized to represent a major antagonist toward improved outcomes for transplanted livers. Attenuation of this deleterious milieu and "resuscitation" of these grafts with the use of *ex vivo* techniques will be the focus of this review [16–18].

Key Points

- Marginal or Extended Criteria Donor (ECD) Livers represent a pool of organs in which recipient outcomes may be suboptimal. As the waiting lists for liver transplantation grow worldwide, increasing reliance on these allografts is crucial
- Liver preservation has been essentially unchanged for more than two decades. Exciting novel work in the area of *ex vivo* techniques such as machine preservation and persufflation of the liver has gained attention of the liver transplant community and has recently moved into the clinical setting
- *Ex-vivo* techniques may "resuscitate" damaged or marginal liver allografts by diminishing preservation related ischemia/reperfusion injury (IRI) thereby improving early allograft function and reducing transplant complications
- Machine perfusion is a platform on which *ex-vivo* therapies such as advanced pharmacologic, stem cell or genetic interventions may be developed

Marginal livers provide an excellent template from which to study the efficacy of preservation techniques

What constitutes a marginal or poor quality liver graft?

Introduced by Renz *et al.*, [10] the term extended donor criteria (EDC) codified utilization of defined types of marginal liver grafts to reduced waitlist mortality. These EDC (= ECD) livers were frequently considered unusable based on retrospective data indicating poor outcomes after transplantation [19]. The novelty in this study was that it highlighted the concept that waitlist mortality could not be thought of in the abstract and that it was a central variable in the analysis of outcomes. Considering that waitlist mortality is an endpoint in survival analysis, ECD donors may offer the possibility of slightly reduced graft survival, but the patient survival is nevertheless improved. ECD livers were defined from donor characteristics that were previously identified to lead to a higher risk of poor early graft function (PEGF) or primary non-function (PNF) which included: age >65 years, [20,21], serum sodium >155 mEq/dl [22,23], macrosteatosis >40% [24,25], cold ischemic time (CIT) >12-h [26], split-liver grafts [27], DCD grafts [28], positive infectious serology, and donors with Centers for Disease Control and Prevention (CDC) high-risk behavior. However, as the ECD livers became more widely utilized, many prominent centers began to define these marginal livers in some variation and extol upon their equivocal outcomes in select patient groups.

At the University of California Los Angeles (UCLA) semantically using the more accepted term ECD instead of EDC, donors were characterized as having an age >50, donor hospital stay >5 days, serum sodium >155 mEq, CIT >12-h and a warm ischemia time (WIT) >40 min [29]. Interestingly, in their experience the usage of livers with 2 or fewer ECD characteristics yielded one-year survival of 77–87% [30]. At the University of Indiana these criteria were broadened to include BMI >34.9, a maximum alanine aminotransferase (AST)/aspartate aminotransferase (ALT) >500 and a maximum bilirubin >2.0 mg/dl at which time they demonstrated comparable 2-year 78–83% overall survival between ECDs and standard criteria donors (SCD) [31].

Historically, these definitions and various adaptions were used to establish standards for what was an innovative use for these marginal livers. However, given that there is no universal definition for an ECD liver, transplant centers are constantly tailoring and altering this classification as their experience dictates. As such, it has often relegated analysis to single center practice or made comparison among multiple centers difficult. Some generally agreed upon donor criteria, which impart risk of adverse recipient outcomes are found in Table 1.

The donor risk index has been used to define marginality in liver transplantation

The introduction of the donor risk index (DRI) by Feng *et al.* has established that there is an increased relative risk of early allograft dysfunction and decrease graft survival with the use of marginal livers [32]. The six donor characteristics found to be significant in multivariate analysis that make up the DRI are: age, DCD, split/partial status, donor race, height, and cause of brain death. Of note, the omission of steatosis from the DRI is a significant problem noted by many clinicians. Nevertheless, validated by the study of a large European cohort, the DRI was found to

Table1. Donor characteristics which impart risk of adverse recipient outcomes.

Elderly donors (>65 yr)	More susceptible to ischemic endothelial injury Decreased ATP availability on reperfusion Less tolerant of prolonged cold ischemia May have decreased synthetic function and regenerative capacity
Underlying liver histopathology	Macrosteatosis → predisposes to early allograft dysfunction and primary non-function Ischemic changes/necrosis Significant alcohol abuse → steatohepatitis Hepatitis B and C activity/portal inflammation Fibrosis → may be associated with hepatitis C or alcohol abuse and may affect long-term outcomes
Ischemia associated with donor injury	Donation after cardiac death → frequently profound ischemia injury High-dose vasopressors Prolonged or uncorrected hypoxemia or acidosis
Biochemical changes	Hypernatremia Rising transaminases or bilirubin

be the strongest predictor of graft functional outcomes [33]. However, examination of national patterns demonstrates an increasing usage of livers with higher DRI scores due to the utilization of grafts from older donors and DCDs in an attempt to curb waitlist mortality [34].

Some recent data demonstrates that the current strategy of offering these livers to patients with lower MELD scores may be counter-productive [35]. As such, the transplant community is at a crossroads as there are clearly patients with decompensation that are underserved by the current MELD classification system [36]. One solution to this predicament that shows promise, is to reduce the IRI insult seen with many marginal livers with the evolving technology of hypothermic machine perfusion (HMP), subnormothermic machine perfusion (SMP), normothermic machine perfusion (NMP), and PSF (persufflation).

Long cold ischemic times may also lead to a poorer quality graft due to derangements in metabolism

Livers subjected to increased CITs are considered marginal grafts due to the obviously heightened risk of PEGF. In trying to explain the degradation of graft function with prolonged CITs, the study of mitochondrial function moved to the forefront after a series of experiments expounded on their role in promoting metabolic and cellular disarray. It was well accepted that ECD/DCD livers are more susceptible to cold preservation injury, specifically with regard to the disruption of oxidative phosphorylation and the possibility of reversing the destabilizing effects. Studying the mitochondrial respiratory function, Kim *et al.* demonstrated that there was a gradual, but pronounced dysfunction in the homogenate of rat livers that underwent extended cold storage (CS) [37]. Interestingly, this poorer mitochondrial function was found to be abrogated in HMP of rat livers up to 48 h [38]. As such, negative effects on the electron transport chain (ETC) were thought to lead to the cellular deterioration seen in prolonged CS.

Classically revered as the *sine qua non* of energy metabolism, the ETC couples adenosine triphosphate (ATP) synthesis to oxygen uptake and allows energy production through the mitochondria membrane to the rest of the cell. Mitochondrial energy derangement leads to severe implications in cellular and organ function if the injuries are widespread. The loss of ATP homeostasis in CS livers has been associated with metabolic and structural changes that can lead to irreversible hepatic parenchymal injury and PEGF [39]. While, the mechanisms are not completely understood, depletion of mitochondrial ATP can lead to profound consequences that include hepatocyte ischemia and necrosis. *In vitro* experiments have shown that ATP diminution causes opening of transmembrane gated proteins on the mitochondrial surface responsible for maintaining a membrane potential. Termed the membrane permeability transition (MPT) pores, the lack of ATP severely impacts their functionality and can ultimately trigger cell death through apoptotic pathways [40].

One hypothesis to explain this mitochondrial uncoupling of ATP synthesis and oxygen uptake in the hepatocyte was expounded upon after an elevated presence of fatty acids was noticed to be associated with this dysregulation. Free fatty acids accumulate in preserved livers and thereby increase oxygen consumption at the expense of a decrease in the content ATP [41]. Importantly, this effect was shown to be countered by short term oxygenated perfusion *in vitro* rodent models [42].

Steatotic livers exhibit impaired ATP synthesis from disruption of the ETC and increased generation of reactive oxygen substances through lipid peroxidation

Steatotic livers are marginal allografts with high levels of PNF and PEGF when implanted [43]. As a result, many of these livers are discarded, especially if the biopsy reveals macrosteatosis >30–40% [44]. Interestingly, the poorer outcomes associated with transplantation of steatotic livers are theorized to be multi-focal, but are centered around impairment of the ETC and ATP synthesis [45]. It is hypothesized that excessive cytoplasmic fatty acids may lead to increased lipoperoxidation yielding more free radicals [46]. This can in turn lead to damage of the cellular architecture and inappropriate Kupffer cell activation with concomitant pro-inflammatory upregulation [47]. The promise of HMP is that it may counter these deleterious effects in the short-term and lead to better long-term outcomes.

Hypothermic machine perfusion

The early history of liver hypothermic perfusion techniques and its evolution

In 1956, Goodrich *et al.* established that WITs as short as 30 min could render the liver untransplantable [48]. Recognizing the limitations imposed by the profound effects of ischemia, Starzl *et al.*

were the first to introduce the concept of hypothermic perfusion as a method to preserve the liver for transplantation by *in situ* cannulation and flushing of the liver [49].

It was in this spirit that the idea of machine perfusion was conceived, when in 1963 using an adaption of the heart-lung machine, cadaveric *in situ* hypothermic perfusion was introduced [50]. While these methods ultimately cooled the liver, clinicians were still hampered by the relatively short allowance of 2–3 h of CIT before transplantation. In an attempt to allow for extended CITs, in 1967 the concept of extracorporeal/*ex vivo* HMP was presented in which 24-h of hypothermic perfusion was demonstrated in canines [51]. Interestingly, this presentation was not considered proof of principle as the donor grafts were implanted into the neck and the canine remained unhepatectomized. Nevertheless, this publication was the first concept of using HMP for liver transplantation. Shortly thereafter in 1968, Brettschneider *et al.* used HMP with homologous blood perfusate and hyperbaric oxygen to push preservation times to 8 h with subsequent canine orthotopic transplantation [52].

Others studies would follow that began to show a crude refinement of this technique, however, they would be short-lived and never reach clinical utilization. The additional cost and complexity of the bulky HMP equipment would be one reason why this nascent technology fell out of favor with clinicians [53]. Moreover, the expanding role and simplicity of static cold preservation solutions that could seemingly protect the liver from ischemia and reperfusion injury gained mass appeal. The advent of Collins/Eurocollins solutions and later University of Wisconsin (UW) solution, gave transplant practitioners a less expensive, reproducible, and simpler means of preserving livers for transplantation [54,55]. As such, research regarding liver HMP would slow for some time. However, the early 1990's would bring about an interest in renal HMP due to the problem of high rates of delayed graft function (DGF) in ECD kidney transplantation. This work has also led to a renewed interest in liver HMP given the greater use of ECD liver grafts.

Metabolic preservation of hypothermic machine perfused livers is profound

HMP ensures homogeneity in the continuous supply of metabolic substrates and antioxidants to the liver parenchyma during the *ex vivo* period. Aerobic respiration is reduced by hypothermia but does not entirely cease. Provision of high-energy metabolic substrate reduces cellular insults typically seen with reperfusion. HMP not only augments the scavenger role of the antioxidants, but also provides a dilutional and washout effect, thereby preventing the accumulation of toxic metabolites and waste products from direct endothelial and parenchymal contact.

Kamada *et al.* described using a continuous pulsatile perfusion technique with fluorocarbons in rats to prolong CITs [56]. Not long thereafter, HMP in a larger animal model was shown to lengthen storage times [57]. For the next two decades, researchers would use these models and others like them to uncover the pathways that became deranged after liver procurement.

Hypothermic machine perfusion could attenuate the deregulation of the oxidative phosphorylation pathway

HMP can lead to abatement of ATP depletion in ischemic livers by sustaining oxidative phosphorylation. As such, comparing ATP concentration in CS and HMP, Dutkowski *et al.* demonstrated that HMP livers provided a greater ATP content at 10-h as compared to CS livers [58]. A similar study performed years later supplemented the understanding of hypothermic perfusion and its impact on cellular viability after prolonged ischemic times. As such, a brief 3-h period of HMP after a 10-h CIT demonstrated no changes in lipid peroxidation, energy charge or increases in cellular injury markers, which included lactate dehydrogenase (LDH) or caspase activity when compared to freshly resected livers [59].

In order to mimic marginal liver grafts and assess the effect on metabolic function, Stegemann *et al.* cold stored rat livers for 22-h before a brief warm ischemic challenge [60]. Subjected to 90-min of HMP, these livers demonstrated an increased energy charge (EC) of ATP phosphorylation as calculated according to Atkinson *et al.* (EC = (ATP + 1/2ADP)/(ATP + ADP + AMP)) [61]. Moreover, as compared to the 22-h CS livers, the HMP livers showed improved vascular resistance, and increased bile production, ammonia clearance and oxygen utilization.

While the mechanisms of this ATP loading remain obscure, perhaps the lower energy demand of a cooled liver and the continuous supply of oxygen may favor this positive ATP balance. As such, this provided the early evidence that HMP technology could lessen preservation injury.

Reactive oxygen substances accumulate during ischemia can also lead to significant hepatocyte toxicity that can be ameliorated by hypothermic machine perfusion

Generally, the mitochondria redox reactions generate relatively few reactive oxygen substances (ROS) as the electrons from nicotinamide adenine dinucleotide (NADH) oxidation are used to create water (H_2O) from oxygen (O_2) and hydrogen (H+) [62]. Moreover, anti-oxidant defenses are thought to play a crucial role in the reductive capacity of these protective cellular mechanisms [63]. Nevertheless, even under homeostatic conditions low level of electrons escape from the downstream redox proteins in the ETC and can create ROS [64].

Under stressful conditions like IRI this process can be exacerbated as the ETC is highly reduced and there is a relative lack of oxygen resulting in the unintended transfer of electrons and creation of ROS [65]. Importantly, Dutkowski *et al.* would provide further evidence that HMP could protect the liver from these ROS; notably there was a decrease in glutathione depletion and superoxide anion release in machine perfused rodent livers as compared to their CS counterparts after 10-h [66]. This is particularly important as it is known that ROS may incite oxidative injury and impact cell viability especially in energy depleted hepatocytes [67]. From these collective experiments it has been deduced that HMP can provide a two-pronged approach by fending off oxygen radical formation and increase radical scavenger activity through glutathione salvage.

Restoration ATP synthesis in steatotic livers to prevent ROS lipid peroxidation in hypothermic machine perfused livers

A study by Bessems *et al.* demonstrated that livers from methionine-choline fed rats resulting in 30–60% steatosis were better preserved if they underwent HMP as opposed to CS [68]. As such, ammonia clearance, ATP levels, urea, and bile production were higher in these steatotic livers that had 24-h of HMP as compared to standard CS.

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The clinical application of hypothermic machine perfusion in livers shows the possibility of changing the landscape of marginal liver utilization

Although, successful LT has being reported after CITs approaching 24-h [69], it is well known that the liver functionality declines after 12-h of cold ischemia and even earlier in marginal livers [70]. For this reason, the extension of HMP into clinical LT has finally gained traction. Moreover, its efficacious use in other organ systems has further spurred the application and development of this technology.

The push of HMP has really been in the clinical experience in kidney transplantation. Over a decade ago, HMP was initially shown to be associated with improved early and long-term renal function as compared to standard CS [71]. More recently, these results were validated in a randomized control trial study that demonstrated an adjusted odds ratio of 0.57 comparing HMP to CS for reduction of DGF and overall graft survival at one year [72]. The three year assessment continued to demonstrate this improved graft survival for HMP kidneys, especially in those from donors after brain death (DBD) [73]. This success story has further fostered the translational aspect of liver HMP as it enters the clinical setting in an attempted rehabilitative effort of poorer quality livers.

Hypothermic machine perfusion of DCD livers offers protection from IRI cellular injury

While DCD donation in kidney transplantation has yielded positive returns, LT of these organs can be fraught with unacceptably high rates of ischemic cholangiopathy and PEGF leading to diminished long-term graft survival. The outcomes associated with organs procured from DCD donors are due to prolonged WITs and low pulsatile flow as the cardiac output begins to profoundly lessen until asystole. As such, DCD livers encapsulate for many centers the very definition of a ‘‘marginal’’ liver and are accordingly frequently declined by most centers. Safe expanded use of these livers could represent a significant viable donor pool, thereby alleviating some of the burden of waitlist mortality.

Beginning at the turn of this century, efforts to resuscitate these livers were centered on the use of HMP in the laboratory. In DCD rodent models, the rehabilitatory effects of HMP could be seen in livers subjected to 30-min of WIT [74,75]. As such, reduction of portal vein pressures and LDH levels were seen after 10-h of perfusion in HMP livers as compared to CS. Likewise, CS livers had larger patchy areas lacking flow as measured by the dearth of fluorescein isothiocyanate-labeled albumin, in contrast to the HMP livers that had more normal flow homogeneity.

Dutkowski *et al.* further advanced the notion that HMP could resuscitate marginal DCD livers with only 1-h of perfusion after 45-min of WIT [76]. Comparing 2 groups of rat livers with 45-min of WIT followed by either 5-h of CS *vs.* 5-h of CS and then 1-h of HMP, the later arm was found to have significantly reduced necrosis after 3-h of normothermic reperfusion. Moreover, only 1-h of HMP after CS seemed to reduce hepatocellular ischemia as seen by decreased AST release and lactate levels in the reperfused livers. Interestingly, livers that underwent HMP for an hour also had less oxygen uptake than non-HMP livers after reperfusion, which in theory could mitigate exposure to oxygen and possible free radical injury.

In a recently published pivotal study, Dutkowski *et al.* reaffirms the importance of oxygenated perfusion for DCD livers [77]. Perfusing through the portal vein using hypothermic oxygenated perfusion (HOPE), another moniker for HMP, oxygen is the limiting factor in reduction of the NADH and a safeguard of mitochondria integrity. Contrastingly, replacing oxygen with nitrogen in this system allows for a highly reduced ETC, Kupffer cell activation, and mitochondrial injury. While these pathways represent a significant obstacle in the successful use of ECD/DCD livers, recently there has also been a renewed interest in the immunologic up-regulation due to warm ischemia.

HMP for a brief period before subsequent CS was found to decrease the release of intracellular adhesion molecule 1 (ICAM-1) and major histocompatibility complex (MHC) class II HLA antigens [78]. Important in facilitating leukocyte endothelial transmembrane migration, ICAM-1 can promote early rejection, while MHC class II overexpression in mismatched donor-recipient pairs may augment an immunological response [79,80]. As such, short term HMP may also protect and ultimately allow immunologic recovery of quiescence in DCD livers. However, while this study has been limited, ongoing work may verify this beneficial mechanism of HMP to marginal livers.

In recent work Schlegel *et al.* also described a protective effect on the rodent biliary system using HMP in DCD grafts that underwent transplantation [81]. Expectedly, perfusion with the HOPE system for only 60-min after 4-h of CS decreased the parameters of hepatocellular injury, lowered the level of DAMP signaling (HMGB-1) and tamped down immunogenic upregulation (CD68, $CD4^+$ T cells). 4-weeks after transplantation, bilirubin levels in these DCD livers perfused with HOPE had a mean bilirubin of 9 μmol/L, while those livers that solely underwent 4-h of CS had a mean bilirubin of 15 μmol/L ($p = 0.08$). While this parameter did not rise to the level of statistical significance, microscopy demonstrated significant cholangiopathic proliferation and fibrosis (Sirius red, α-SMA, CK-19) in the untreated DCD liver group as compared to those that underwent HMP.

The realization of hypothermic machine perfusion in transplantation

Notwithstanding the interesting findings associated with these *in vitro* and small animal studies, discovery as it would pertain to liver recovery and reconditioning largely found its place in the porcine model. Studies by Mastuno and Uchiymama would provide support for the use of swine livers to study HMP and rehabilitation because of technical feasibility of both perfusion and transplantation [82,83]. In 2009 de Rougemont *et al.* were amongst the first to show that a short run of HMP could rescue pigs from certain death when undergoing DCD transplantation [84].

The early results from human trials with hypothermic machine perfusion highlight the future possibilities

Earlier work in animal and human discard liver models facilitated the translation of HMP into a human clinical trial for the first time by our group [85]. Guarrera *et al.* at Columbia University became the first investigator worldwide to use HMP on human livers that were transplanted successfully [86]. The purpose of the initial trial was to assess its reliability and safety of liver HMP in a clinical setting. A modified Medtronic (Minneapolis, MN) non-pulsatile pump and Vasosol® solution was used to perfuse

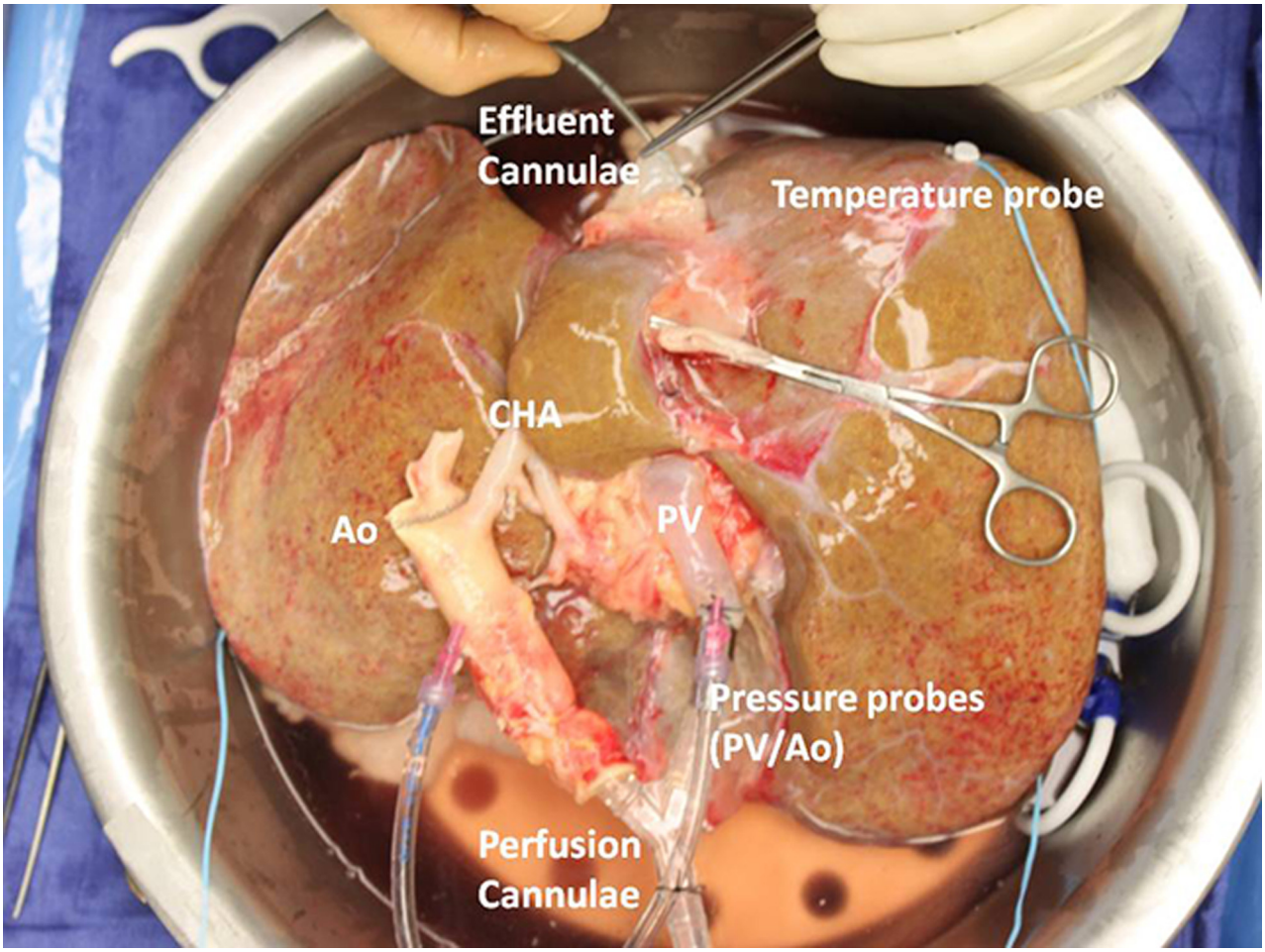

Fig. 1. A representative liver allograft undergoing HMP from the Columbia University Medical Center Liver HMP Clinical Trial (ClinicalTrials.gov: NCT01274520). PV, Portal vein; Ao, Aorta; CHA, Common hepatic artery.

20 transplants in a case-controlled clinical trial of liver HMP in humans. HMP was associated with decrease in early allograft dysfunction (as defined by the Clinical Trials in Organ Transplantation Study Group) compared to those in CS control group [87]. In addition, HMP livers exhibited lowered IRI markers, peak transaminases, improved renal function, and shorter hospital length of stay. A representative liver allograft undergoing HMP from our trial is shown in Fig. 1.

Immunohistochemistry and RT-PCR analysis of livers in this phase I clinical trial was assessed for the post reperfusion expression of pro-inflammatory cytokines and adhesion molecules. As would be predicted, the livers from the HMP arm showed attenuated chemotactic cytokine over-expression and thereby prevented leukocyte recruitment and Kupffer cell activation along the donor endothelium [88]. The cellular and cytokine injuries specific to the liver are succinctly summarized in Fig. 2. In addition, effluent analysis during HMP showed a reduction in these acute phase reactants. This reduction was also shown to be sustained and progressive on the HMP device [89].

Of note, a US Health Resources and Services Administration–sponsored trial using HMP to preserve ECD livers has been completed at our center with promising results including shorter length of stay, better early liver and renal function and significantly less biliary complications. [16]. More recently, Dutkowski *et al.* advanced the applicability of HMP by using an ECOPS device (Organ Assist®) to perfuse 8 DCD donor livers (Maastricht category 3) prior to transplantation [90]. After a strict 10-min "no-touch" period following asystole, using a double balloon catheter in the iliac artery the donor organs were flushed and retrieved with a functional mean WIT of 31-min. The liver was subsequently placed on the HOPE system and transplanted after 1–2 h of reperfusion at 10 °C. Importantly, hepatic function was comparable with DBD donor livers after transplantation and there was no evidence of biliary cholangiopathy on MRCP. This study represents the first foray into the human clinical use of HMP for DCD livers and dramatically raises the bar for the possibilities and utility of this technology.

The human discard liver HMP experience would reinforce the benefits seen in the clinical setting

The arrival and success of these clinical human trials would provide the backdrop for adjunctive studies that would test the limits of the applicability of HMP technology. Generally reflecting the poorest quality livers because of severe macrosteatosis or DCD status, livers that were unable to be allocated were used

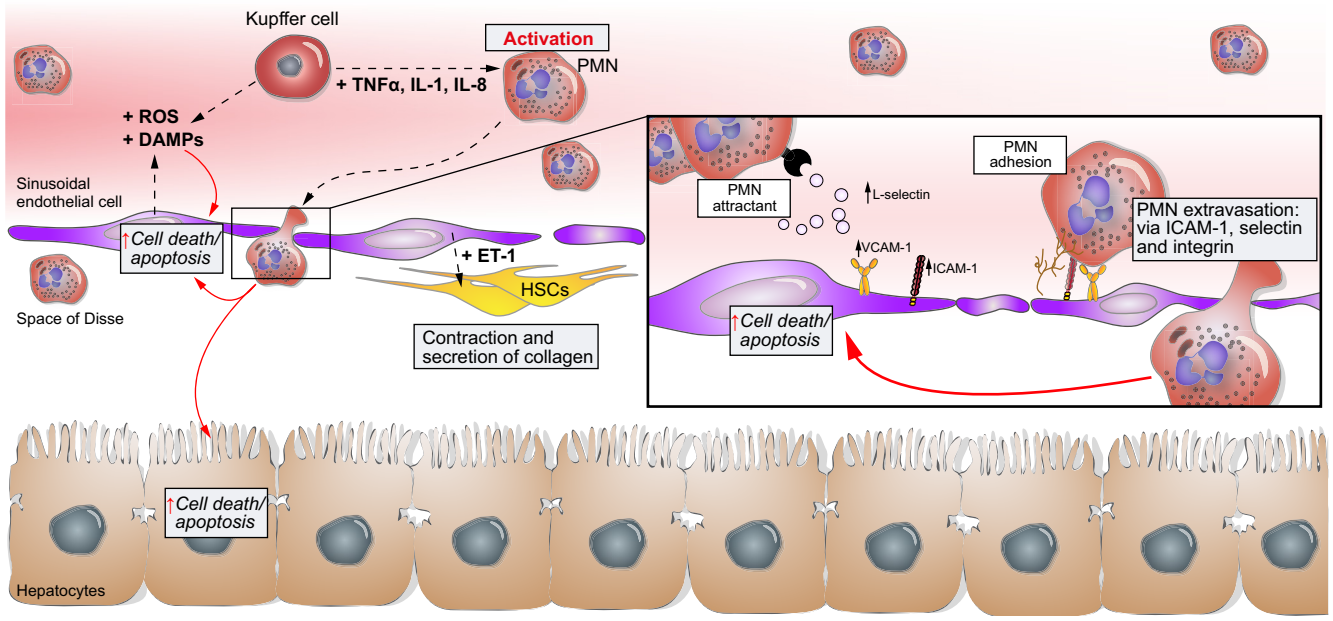

Fig. 2. Liver specific cellular and cytokine activation and injuries associated with transplant ischemia/reperfusion injury.

in a HMP discard model by several investigators to test efficacy of HMP even in the extreme. Vekemans *et al.* applied HMP to 12 human discard livers with oxygenated flow-limited perfusate through the portal vein (3 mmHg) and common hepatic artery (20 mmHg) [91]. After 4-h of HMP, these livers displayed improved hepatocellular injury metrics (AST, LDH) during rewarming as compared to a CS cohort of discarded livers. Investigators have also promoted the use of HMP as a means to indicate the liver quality prior to transplantation. Again using a human discard model, Monbaliu *et al.* dichotomized discarded livers into two groups; not transplantable livers (macrosteatosis >30%, toxic hepatitis with cholestatic appearance and alcoholic steatohepatitis) and potentially transplantable livers (allocation problems) [92]. Not unexpectedly, perfusate from the non-transplantable group had higher levels of AST and LDH despite HMP. In this way, HMP may allow for efficient graft evaluation as differences in these biomarkers became readily apparent as early as 30-min after the commencement of HMP.

Perfusates for machine perfusion

Collins solution, blood components or UW solution were initially used in earlier liver HMP studies. The optimal perfusate has not yet been determined and is a topic of debate. Our clinical liver HMP trials have all used Vasosol® with promising results. Vasosol® is based on Belzer's Machine Perfusate which is utilized for Renal HMP (KPS-1, Organ Recovery Systems, Itasca, IL, USA), Vasosol® is enhanced with alpha-ketoglutarate as an energy substrate and mitochondrial stabilizer, nitroglycerine and L-arginine as a nitric oxide precursor, N-acetylcysteine as a glutathione precursor, and both nitroglycerin and prostaglandin E1 as vasodilators. Our lab is currently investigating perfusates and has shown benefits of Vasosol® over Belzer MPS™ in a rodent model of HMP. We also saw a benefit in adding alpha tocopherol (Vitamin E) to Vasosol® to further enhance antioxidant properties (unpublished data, in submission).

Specific perfusate requirements will also vary based on the temperature of machine perfusion. Normothermic perfusion requires advanced metabolic support since the organ is fully metabolically active. NMP has typically utilized diluted blood based perfusates and more recently the acellular "Steen Solution".

Polysol™, a balanced solution enriched with various amino acids, vitamins, antioxidants (alpha-tocopherol, ascorbic acid and glutathione), impermeants (raffinose and trehalose), and other metabolites (glucose) [93]. In rodent studies, HMP livers pumped for 24 h in Polysol™ demonstrated less cellular damage as indicated by assessing and noting the lessened serum spillage of liver functional enzymes. Polysol™ was then evaluated in rodent livers subjected to a pre-HMP 30 min WIT to mimic a DCD organ [94]. After 24-h of perfusion, the livers were reperfused with Krebes-Henseleit buffer. Again there was less AST and ALT leakage when comparing HMP-Polysol™ livers to CS or UW-livers. Moreover, there was less readily apparent vacuolization, sinusoidal edema or necrosis seen in the HMP-Polysol™ organs.

Normothermic machine perfusion

Is the field of liver preservation warming up?

More recently, the concept of hypothermia as a necessary entity to decrease the metabolic requirements of the liver has been challenged. Normothermic preservation may lead to sustained viability, improved hemodynamics and attenuate ischemic injury in marginal livers [17]. An *ex vivo* liver perfusion system was initial used to investigate NMP [95]. These livers were reperfused with porcine blood at 32 °C or 37 °C after a period of cold

preservation with varying results. Friend *et al.* would also be amongst the first to describe NMP and its benefits over standard CS as measured by preservation of ALT and enhanced Factor V production [96]. Minor *et al.* demonstrated the rewarming of porcine liver grafts up to 20 °C even over a short course resulted in reduced enzymatic leakage, lipid peroxidation, and histologic injury [97]. However, it may be better to never cool the liver and continue NMP, thereby avoiding a hypothermic period that may have a deleterious effect on the graft [98].

In another study, the blood based perfusate was rewarmed to 37 °C and perfused through discarded human livers for 6-h [99]. This study represents the first foray of NMP into studying human grafts and challenges that were met. Importantly, these livers demonstrated both increased biochemical viability and decreased hepatocellular injury. Using this whole blood perfusate at 39 °C, Imber *et al.* would also show that porcine livers were able to maintain normal bile production for 20-h with supplementation with taurocholate [100]. Glucose metabolism and galactose clearance were also corrected with 24-h of NMP as compared to CS [101]. Moreover, using this model, NMP for an astonishing 20-h after 40-min of WIT significantly increased post-transplant survival [102].

Injury from warm ischemia in DCD livers may be attenuated using normothermic machine perfusion

St Peter *et al.* also demonstrated that porcine DCD livers with 60-min of WIT that underwent NMP faired significantly better than standard CS [103]. Notably, ALT levels were markedly lower in NMP livers than CS controls after reperfusion, suggesting a much lower degree of hepatocyte cytolysis. Moreover, histological specimens from the livers in the CS arm confirmed the presence of gross hepatocellular necrosis and sinusoidal hemorrhage while the NMP were relatively sparred. Accordingly, they suggest that DCD livers maybe rehabilitated after a course of NMP. In another animal model designed to mimic logistics of actual procurement practices, rat livers were exposed to 45-min of WIT followed by a 2-h period of CS to mimic the initial procurement and transport logistical period [104]. These livers were then placed on the NMP circuit for 4-h and then transplanted with resultant 100% 1-month survival.

Transferring DCD livers after 60-min of WIT immediately to the NMP circuit may be more beneficial than cooling the liver for 1-h in CS solution prior to NMP (CS + NMP) [105]. While CS + NMP livers showed similar synthetic and biliary production characteristics as compared to NMP alone livers, hepatocellular damage as measured by AST, beta-galactosidase (Kupffer cell injury), and hyaluronic acid (endothelial cell impairment) was more apparent. As such, NMP may attenuate the CS injury to the endothelial cell as described by Ikeda *et al.* transplanted rodent livers [106].

In an important paper by Schön *et al.*, the term normothermic extracorporeal perfusion (NELP) was coined to describe NMP [107]. Again utilizing whole blood mixed with an isotonic solution, donor livers were perfused with the NELP circuit at 37 °C. Importantly, NELP allowed for survival in all transplanted grafts and facilitated recovery of DCD livers exposed to 1-h of WIT. Tolboom *et al.* also similarly showed the rehabilitatory properties of NMP when DCD Lewis rat livers exposed to 1-h of WIT had a 92% 4-week survival if they were placed on the NELP circuit for 6-h prior to implantation, while non-NELP livers all died within 12-h [108]. This experience was repeated, albeit with a more extreme time frame, when Butler *et al.* placed porcine livers on the NELP circuit for 72-h [100,109]. While non-NELP livers showed massive necrosis after 72-h, those undergoing NMP had preserved architecture and synthetic function.

Ex vivo NMP in DCD porcine livers at 38 °C may also facilitate sustained oxygen extraction with normalized metabolism [110]. ATP is likewise restored to pre-ischemia levels despite WIT of 1-h with NMP after cardiac arrest in Yorkshire pigs [111]. During the warm ischemic phase in DCD grafts, ATP can rapidly be depleted [112]. As such, this extracorporeal NMP system with porcine blood was able to reconstitute a more normal hepatic milieu after only 4-h of perfusion.

Extracorporeal membrane oxygenation allows for in situ normothermic perfusion of marginal livers

Initial reports of donation after prolonged extracorporeal membrane oxygenation (ECMO) in otherwise standard criteria donors lead to the consideration of an expanded role for this technology [113,114]. Cannulating a major artery and vein, ECMO offers *in situ* normothermic perfusion before procurement, which can counter the disastrous effects of WIT after cardiac arrest. As such, Rojas *et al.* began some of the first experiments to assess the use of ECMO in DCD swine with assessment of liver markers [115]. The appearance of this procedure in the clinical setting would be rapid, when a combined group of practitioners at Michigan and Wisconsin would report the use of ECMO in DCD donors for kidney transplantation [116]. Following this report, Jiménez-Galanes *et al.* would describe the first prospective human study of ECMO in uncontrolled DCD donors (n = 20) in liver transplantation [117]. When compared to DBD LTs (n = 40), the one-year graft survival was equivocal (87.5% *vs.* 80%), although 15% of patients in the ECMO DCD arm would ultimately need to be re-transplanted.

While this experience is rare in the United States due to existing donor laws, in Spain, family consent is not legally needed in order to start ECMO in Maastricht 2 donors, in which death is unpredictable [118]. Therefore the Spanish DCD practice largely reflects the utilization of asystolic trauma victims that have undergone unsuccessful resuscitation by the first responders and have had a 5-min of a "no-touch" period, after which time the donor can be placed on the ECMO circuit. Generally the femoral artery and vein are cannulated with supra-celiac occlusion balloon placement [119]. Of note, due to the clinical nature of these studies the WIT accrued is *in situ*, while the previous animal models allow for WIT *ex vivo*. It is still unclear if these differences in WIT can affect the liver with varied manifestations.

In a novel approach, Fondevila *et al.* used ECMO (termed normothermic extracorporeal membrane oxygenation or NECMO in this manuscript) for 60-min after a sustained period of cardiac arrest (90-min) in donor swine [120]. After NECMO, the livers in this study either had CS or NMP; NECMO + NMP had decreased pro-inflammatory markers, like IL-6, TNF, and vWF as compared to the NECMO + CS group.

While the transplant community has not adopted the use of this NMP in any clinical setting as of yet, it represents a new foray into preservation and may ultimately yield important dividends for optimizing marginal grafts. Moreover, NMP will likely facilitate an expanded scientific query into the best composition of normothermic perfusate, given that whole blood components

have been used commonly and have significant drawbacks. There are also many technical concerns with NMP. If monitoring is inadequate or the device fails there is rapid warm ischemic injury to the allograft. There are also concerns around microbial contamination and transmission to the recipient that needs to be further addressed.

Subnormothermic machine perfusion

Subnormothermic machine perfusion has also been explored as a means to reconditioning marginal livers

More recently, SMP has been advocated as a possible alternative. Olschewski *et al.* suggested that perfusion at 21 °C for 6-h through the portal vein of Wistar rat livers after a 1-h WIT (DCD) was better than with temperatures of 4 °C or 12 °C [17]. To reenact *in vivo* reperfusion, all livers were then perfused at 37 °C. Livers subjected to SMP at 21 °C demonstrated reduced portal pressures, better bile production, and decreased markers of ischemia. In a porcine model, steatotic livers studied with SMP maintained factor V and bile production comparable to normal livers [121].

Tolboom *et al.* also presented that SMP was beneficial to reconditioning transplanted rodent livers subjected to 1-h of WIT after cardiac arrest [122]. However, while the cooler temperatures may reduce oxygen uptake in this experimental model, the effect on the phenotypical expression of ischemia was a mixed picture. Given the limited research on SMP, the use of this technique is unclear. Moreover, it is not known if promising results reflect the positive effects of machine perfusion on the whole.

Persufflation

The early validation of persufflation as a means for preservation

Remarkably, PSF started by accident in 1902 by Rudolf Magnus when his liquid perfusate supplying a cat heart ran empty and pressurized oxygen gas was drawn into the circuit [18]. To his surprise, Dr. Magnus observed rhythmic pulsations for up to 9-min after the reservoir ran dry. This was the beginning of PSF and the ushering in of subsequent studies illustrating that gaseous oxygen perfusate could allow for contractility from over an hour.

Extrapolating this concept to the liver, Fischer *et al.* initially studied PSF in rat livers with retrograde oxygenated gas perfusion of the hepatic veins and puncturing the liver with "blow holes" to allow the gas to escape [18,123]. Minor *et al.* was the first to validate that PSF might be useful for livers in a transplant setting by infusing gaseous oxygen at 18 mmHg for 48-h at 4 °C into Wistar rat livers [124]. 48-h after PSF, the livers were reperfused in Krebs-Henseleit buffer and were found to have dramatically lower levels of Kupffer cell activation as compared to the non-PSF controls when measuring acid phosphate.

PSF has also been shown to reduce cellular proteolysis in rodent liver grafts [125]. As proteolytic activity has been associated with impaired hepatic injury, inhibition of redox reactions can lead to inappropriate oxidation of intracellular components [126].

Persufflation may rehabilitate marginal livers

Minor *et al.* also established that PSF or venous systemic oxygen persufflation (VSOP) could be used to rehabilitate DCD livers after 30-min of cardiac arrest [127]. Transplanting these livers after 24-h of PSF, there was enhanced NADH re-oxidation, normalized ATP concentration, and sustained ATP energy charge potential. This decreased tissue specific NADH was measured with microscopic fluorescence substantiating the notion of preserved mitochondrial oxidative phosphorylation [128].

Remarkably, PSF was also shown to rehabilitate rat livers after 47-h of CS in UW solution. Attaching these livers to the PSF circuit for only 1-h lead to lower AST levels and conserved ATP concentrations after *in vitro* reperfusion as compared to solely CS livers [129].

PSF was also examined in steatotic livers in a rodent model of starvation followed by fat-free carbohydrate rich diet [130]. Recapitulating moderate steatosis, PSF for 24-h lead to better maintained sinusoidal endothelium and mitochondria integrity. Moreover, again AST levels were lower as well as glutamate dehydrogenase levels, possibly reflecting less parenchymal damage which coincided with decreased portal venous pressures upon *in vitro* reperfusion.

However, while PSF has been able to sustain oxidation metabolism, concerns over oxidative free radical injury to stress parenchyma has arisen. Furthermore in a recent study, superoxide dismutase or *n*-acetylcysteine in conjugation with PSF, lowered the concentration of lipid peroxidation in DCD rodent livers [131]. Interestingly, poorer results were seen without these anti-oxidants, in part supporting the notion that PSF may promote a damaging oxidative milieu in ischemically challenged grafts. After a short course of high-dose reduced glutathione, concomitant PSF can also protect steatotic rodent livers from ischemic damage after transplantation [132]. Moreover, glutathione, a free radical scavenger was shown to reduce hepatic necrosis in pre-treated fatty livers as compared to the non-treated arm, and improve survival.

Nitrous oxide (NO) is yet another compound that has shown promising results in persufflated livers due to its anti-oxidant and vasodilator properties [133]. Srinivasan *et al.* would validate this assertion by persufflating DCD rodent livers with gaseous oxygen and 80 ppm NO via the IVC [134]. Expectedly, upon reperfusion, livers in the VSOP-NO arm had a decreased presence of hepatocellular inflammatory markers and lower levels of malondialdehyde, an indicator of oxidative stress and lipid peroxidation. In the ultimate litmus test, Yagi *et al.* established that VSOP-NO partial rodent liver grafts had better regeneration metrics, higher Ki67 expression and upregulated hepatic endothelial NO synthase (e-NOS) [135]. e-NOS can counter endothelin-1 induced vasoconstriction, bettering the hepatic microcirculation and lessen TNF-α expression [136]. VSOP-NO may also rehabilitate partial liver grafts encumbered with steatosis when measuring the usual parameters of parenchymal damage and graft viability [137].

Larger animal models to establish the efficacy of PSF in these marginal grafts would follow with the use of a porcine system. Again, PSF would prove to be effective in altering in hepatic injury as measured by AST and LDH in DCD grafts [138]. Strikingly, animals transplanting with a DCD liver that did not undergo PSF all died shortly after 3-h of reperfusion. Later it was determined that 2-h was the optimum time of PSF following CS that allowed for

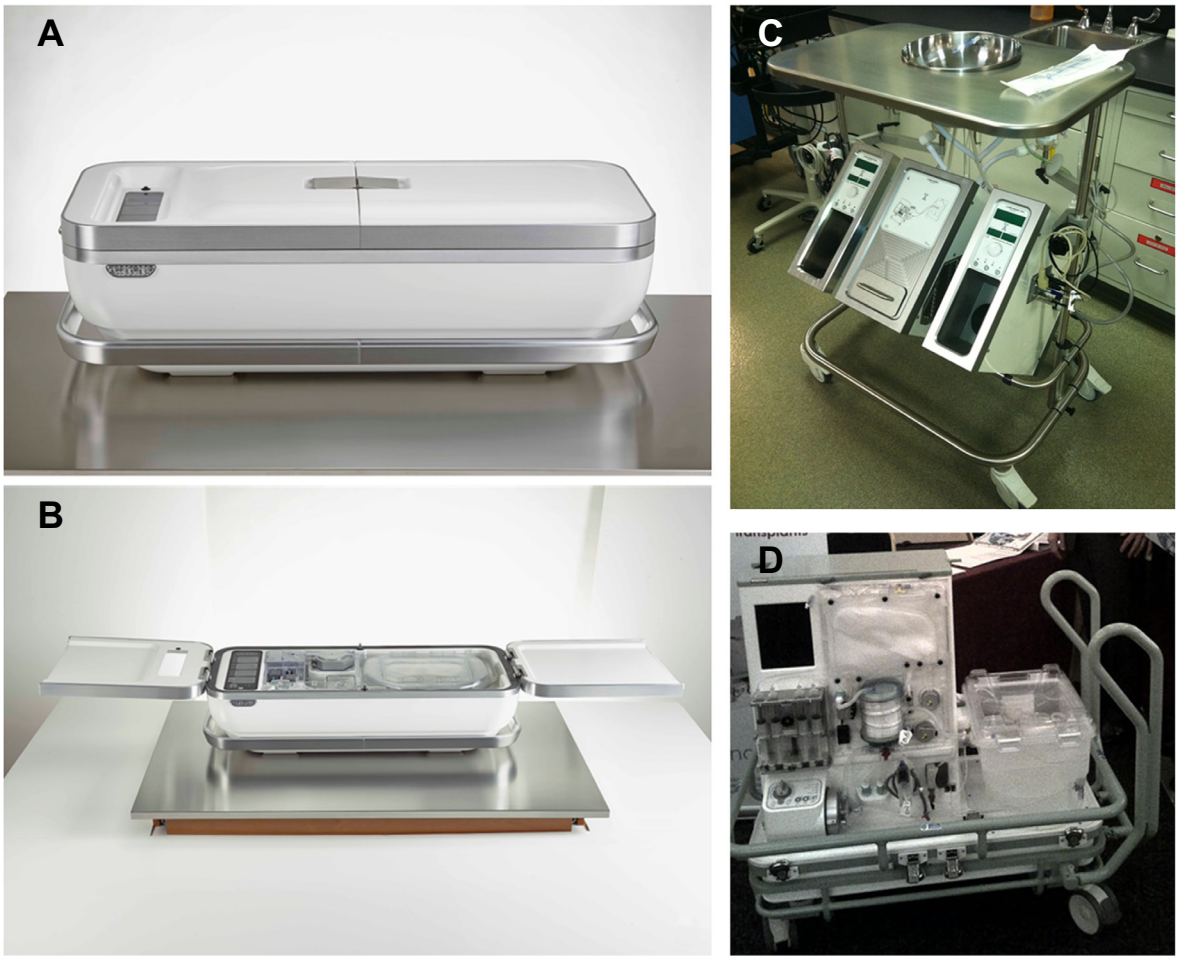

Fig. 3. Machine perfusion devices. (A, B) Photograph of Organ Recovery Systems Inc. LifePort® Liver Transporter prototype. (C) ECOPS device (Organ Assist®). (D) The OrganOx® *metra*™ device.

decreased enzymatic release of AST, LDH, and TNF-α, and which correlated with 83% 7-day survival [139,140]. Portal venous flow parameters in these reperfused grafts were also improved with 2-h of PSF, likely representing less parenchymal edema from IRI.

Persufflation in the clinical setting

The early foray of PSF into the clinical setting took place in 2004 when surgeons in Germany transplanted 5 livers from DCD donors with WITs in excess of 20-min and that had been turned down by at least 3 centers [141]. In this pilot study, all of these livers were persufflated through the IVC for at least 1-h prior to transplantation and biopsies were taken before and after being placed on the PSF circuit. One patient suffered from a hepatic artery thrombosis post-operatively that resolved with thrombectomy and another from a subcapsular hematoma that needed a wash-out. ATP levels in the liver tissue post-PSF were more than 2-fold pre-PSF concentrations.

As such, in 2011 a registered human trial was started to examine in a larger cohort, 2-h of PSF in transplanted livers [142]. Prospective and randomized, this single center study entitled "Oxygen persufflation as adjunct in liver preservation" (OPAL) looks to assess the effects of 2-h of PSF and the primary outcomes of peak transaminase serum levels (AST), early-onset of graft dysfunction, patient and graft survival.

Technological advances in preservation

The development of newer machine perfusion devices will take a concerted effort between medicine and industry

The development of reproducible liver machine perfusion pumps has begun relatively recently. Taking a cue from kidney perfusion pumps, the advent of liver machine perfusion pumps at the basic level included an organ chamber, heat exchanger, membrane oxygenator and a vascular circuitous pump mechanism. The Groningen Liver Perfusion Pump was among the first prototypes described for large animal liver HMP [143]. Relying on a dichotomous oxygenated pumping system that allowed for continues flow to the portal vein (4 mmHg) and pulsatile flow to the hepatic artery (30/20 mmHg), this pump facilitated complete perfusion of porcine livers without evident histological damage. Notably, in earlier studies these pressures were set with regard to concerns relating to endothelial injury. In machine perfusion livers there arose the notion that the normally low-pressure portal venous

system would be unduly affected by small variations in pressures seen during continuous perfusion. Particularly, 't Hart *et al.* showed that rodent livers undergoing HMP fared better with 25% of their normothermic circulation (mean arterial perfusion pressure of 25 mmHg and a portal perfusion pressure of 4 mmHg) as opposed to 50% (mean arterial perfusion of 50 mmHg and 8 mmHg portal perfusion pressure) [144].

A few years later in Belgium, Monbaliu *et al.* described a similar pressure-controlled and flow-limited continuous perfusion pump in a porcine model and calculated the resistance parameters during perfusion [145]. Progressive arterial relaxation as manifested by decreased vascular resistance was seen until a steady state was reached at 6-h after initiation of HMP. Other more recent designs included one machine perfusion system with 3 circulating systems for enhanced heat exchange and an oxygenator of the dichotomous arterial and portal venous circuits [146]. Our current work is progressing rapidly on a more portable and durable liver machine perfusion pump in the form of LifePort Liver Transporter®; Organ Recovery Systems (Chicago, IL) with extensive design input from our group to insure practicality and widespread clinical applicability (Fig. 3).

Conclusion

Despite the history of mixed results with the use of marginal livers, safe utilization of these grafts holds the potential to reduce waitlist mortality and improve access to liver transplantation. While shorter CITs and improved donor selection is a good start, the potential of a wide array of *ex vivo* interventions is now progressing into clinical application. These interventions that change the way we practice medicine by expanding the donor pool and improving clinical results with marginal allografts liver is exciting and undeniable.

Perhaps most importantly, exciting preclinical work using machine preservation as a platform for elaborate *ex vivo* interventions such as knockout of deleterious IRI genes, stem cell therapy, and bio-artificial liver development have been proposed by cutting edge innovators. Such interventions hold further promise for *ex vivo* resuscitation of marginal liver allografts.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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